Magnetic Resonance Microscopy of Collagen Mineralization

Ingrid E. Chesnick,* Jeffrey T. Mason,* Anthony A. Giuseppetti,† Naomi Eidelman,† and Kimberlee Potter*
*Department of Biophysics, Armed Forces Institute of Pathology Annex, Rockville, Maryland; and †Paffenbarger Research Center, American Dental Association Foundation, National Institute of Standards and Technology, Gaithersburg, Maryland

ABSTRACT A model mineralizing system was subjected to magnetic resonance microscopy to investigate how water proton transverse (T_2) relaxation times and magnetization transfer ratios can be applied to monitor collagen mineralization. In our model system, a collagen sponge was mineralized with polymer-stabilized amorphous calcium carbonate. The lower hydration and water proton T_2 values of collagen sponges during the initial mineralization phase were attributed to the replacement of the water within the collagen fibrils by amorphous calcium carbonate. The significant reduction in T_2 values by day 6 ($p < 0.001$) was attributed to the appearance of mineral crystallites, which were also detected by x-ray diffraction and scanning electron microscopy. In the second phase, between days 6 and 13, magnetic resonance microscopy properties appear to plateau as amorphous calcium carbonate droplets began to coalesce within the intrafibrillar space of collagen. In the third phase, after day 15, the amorphous mineral phase crystallized, resulting in a reduction in the absolute intensity of the collagen diffraction pattern. We speculate that magnetization transfer ratio values for collagen sponges, with similar collagen contents, increased from 0.25 ± 0.02 for control strips to a maximum value of 0.31 ± 0.04 at day 15 ($p = 0.03$) because mineral crystals greatly reduce the mobility of the collagen fibrils.

INTRODUCTION

The study of mineralized collagen by conventional techniques, such as x-ray or neutron diffraction, has yielded little information about subtle changes to the collagen matrix that might occur during the mineralization process. Many reports have focused on the effect of drying or demineralization on the collagen lateral packing (1–5), or the effect of different mineral densities, from different tissue sources, on the collagen fiber *d*-spacing (6). Alternatively, scanning electron microscopy (SEM) and transmission electron microscopy have been used to study the arrangement of mineral crystals around collagen molecules (7–10); however, such studies demand that mineralized specimens be exposed to various preparative solvents that can alter the natural spacing of the collagen fibrils. In this work we propose to study a model system in which a collagen sponge is mineralized with calcium carbonate, using magnetic resonance microscopy (MRM). This noninvasive imaging modality is capable of generating parametric maps of water proton transverse (T_2) relaxation times and magnetization transfer ratios (MTRs), which can be used to monitor the state of mineral and collagen, respectively.

Water proton T_2 values are highly dependent on molecular motion; thus, when water molecules become immobilized through ionic or dipolar interactions at the surface of a mineral, the T_2 of the surrounding solution is reduced because of fast exchange processes with the mineral-bound water molecules (11,12). In the presence of mineral deposits with a bulk magnetic susceptibility different from that of water, T_2 relaxation times are further reduced due to heterogeneities in the local magnetic field, and can therefore provide a measure of the mineral content of the sponge. With x-ray-based techniques, this may be difficult to achieve because of the relatively low mineral content. This is particularly the case if the mineral is amorphous, which broadens the diffraction peaks and reduces the ability to resolve them.

Alternatively, one can assess the collagen component of this model by measuring the MTR of surrounding water protons. Typically, macromolecular spins exhibit a much broader absorption line shape than mobile water protons and can be selectively saturated by the application of an off-resonance pulse before a standard imaging sequence (13,14). Saturated macromolecular spins exchange with mobile water protons, resulting in a reduction in the detected signal compared to a sequence without an off-resonance saturation pulse. The amount of signal loss is dependent on the exchange processes that take place within the tissue, and can be used to identify unique tissue components. For example, it is widely accepted that collagen-containing tissues give rise to a significant MT effect, which is the result of cross-relaxation between mobile water protons and hydroxyl groups on the hydroxyproline residues of collagen (13,14). Notably, calibration curves of MTR and collagen content have been derived for articular cartilage (15), engineered cartilage (16), and collagen gels (15,17,18). In our experience, collagen in mineralizing tissues, such as the avian growth plate (19), calcifying cartilage (20), and osteoblast-seeded polymer scaffolds (21), gives rise to a significant MT effect. More importantly, in zones where mineral deposits are localized, the MT effect is enhanced compared to that in surrounding unmineralized tissue (22). However, as the mineral content

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Address reprint requests to Kimberlee Potter, PhD, Dept. of Biophysics, Armed Forces Institute of Pathology Annex, 1413 Research Blvd., Rockville, MD 20850. Tel.: 301-319-0208; Fax: 301-319-0638; E-mail: potterk@afip.osd.mil.

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approaches that of mature bone, the T_2 of the water protons in the bone is markedly reduced and thus the MT effect cannot be measured accurately. We expect that the measured MT effect before mineralization reflects the collagen content of the mineralizing osteoid tissue. We speculate that during bone formation, the MT effect is enhanced due to an increase in the collagen content of the mineralized zone, brought about by formation of mineral between the collagen fibrils and the correlated reduction in the intermolecular spacing of the collagen fibrils (23). The collagen fibrils pack together more tightly, resulting in a higher volume density of collagen and thus higher MTR values.

To characterize the changes we have observed for bone (19–22), we subjected a model mineralizing system to MRM. In this model system, calcium carbonate mineral is produced when an aqueous solution of calcium chloride is exposed to the decomposition products of ammonium carbonate. In the presence of a charged polypeptide additive, such as poly(acrylic acid) or poly(aspartic acid), calcium carbonate is formed as a liquid-droplet mineral precursor phase (24). This polymer-induced liquid-precursor (PILP) is drawn into the gaps and grooves of collagen fibrils by capillary action, and upon solidification forms plate-like crystals within the collagen fibrils reminiscent of those observed for normal bone (25–27). The formation of intrafibrillar mineral deposits has been confirmed by selected area diffraction of the center of a mineralized collagen fiber (27). In the absence of poly(acrylic acid), rhombohedral crystals of calcite form on the surface of the collagen fibers (26,27). To confirm that we were able to reproduce the model mineralizing system described in the literature (25–27), we subjected our model system to SEM. The advantage of this model system is that the quantity of collagen is unchanged, so observed changes in the measured MT effect can be attributed to changes to the collagen fibrils induced by the mineral deposits.

The lateral spacing or equatorial diffraction spacing of collagen derived by x-ray diffraction has been used to confirm the lateral collapse of the collagen present in mineralized turkey leg tendon compared to unmineralized tendon (1,4). Therefore, we attempted to assess changes in the lateral packing arrangement of collagen during the mineralization process by using x-ray diffraction to examine collagen sponges mineralized under PILP conditions. If the behavior of water protons reported by MRM can be related to molecular level changes to the collagen fibrils due to mineral deposition, it is our expectation that the MRM experiment can be used to gain important insights into the mineralizing process in vivo.

MATERIALS AND METHODS

Mineralization of collagen sponges

Details of the PILP process are described elsewhere (25–27). Briefly, a Cellagen (reconstituted Type I collagen) sponge (ICN Biomedicals, Aurora, OH) was cut into rectangular strips (2 mm × 10 mm × 1 mm), and eight strips were prepared for each time point. Before mineralization, the collagen strips were rehydrated in 12 mM calcium (II) chloride dihydrate ($CaCl_2.2H_2O$; Sigma-Aldrich, St. Louis, MO) at 4°C for 3 days. At the start of the mineralization process, four strips were placed in a petri dish (diameter = 35 mm; Corning, Corning, NY) containing 1.5 mL of 24 mM $CaCl_2$, 1 mL of 1 mg/mL polyacrylic acid (PAA, poly (acrylic acid, sodium salt), M_w = 5100; Sigma-Aldrich), and 0.5 mL of deionized water, for a final concentration of 12 mM $CaCl_2$ and 0.33 mg/mL PAA. Two petri dishes containing four strips each were used for each time point. Before use, all solutions were filter-sterilized using a benchtop filter flask (Corning) with a 0.22-μm filter. The dishes were sealed with Parafilm and three pinholes were introduced in the film to allow for the exchange of gases. Dishes were transferred to a dessicator with three vials containing 3 g crushed ammonium carbonate (Sigma-Aldrich) each. The vials were sealed with Parafilm and a single pinhole was made in the film to allow the diffusion of ammonia and carbon dioxide. The dessicator was evacuated and stored at 4°C. Every 3 days the calcifying solution for the existing dishes was changed and additional dishes were added to the chamber. This process was repeated until 24 days had passed. In addition to experimental samples, a control dish was included with each series. The control dish contained the same calcifying solution as the experimental samples (12 mM $CaCl_2$ and 0.33 mg/mL PAA), but the strips were not exposed to the ammonium carbonate.

At the end of 24 days, all of the samples were removed from the desiccator, rinsed twice in 12 mM $CaCl_2$ to remove any residual PAA, and stored in dishes containing 12 mM $CaCl_2$ at 4°C. MRM was performed on at least three sets of samples containing strips for the different time points. After MRM, at least two specimens from each time point were analyzed by x-ray diffraction. One set of strips was used for SEM analysis (see below). The remaining specimens were subjected to a biochemical assay for calcium.

Biochemical assay for calcium

Each collagen strip was washed with deionized water to remove residual calcium chloride before the assay. The details of this Alizarin red-based calcium assay have been published elsewhere (28). Briefly, each sample was placed in a 1.5 mL Eppendorf tube to which was added 250 μL of a 40 mM aqueous solution of Alizarin red S (Sigma-Aldrich). The samples were incubated with shaking for 20 min at room temperature. After the incubation period, excess Alizarin red dye was removed and the samples were washed with 1 mL of deionized water for 5 min. This step was repeated to remove excess dye from the sample. Excess water was removed, 800 μL of 10% v/v acetic acid (Sigma-Aldrich) was added to each tube, and the samples were incubated for 30 min at room temperature. After the incubation period the samples were vortexed for 30 s and overlaid with 500 μL of light mineral oil (Sigma-Aldrich). They were then incubated for 10 min at 85°C to solubilize the Alizarin red-calcium complex. The samples were cooled on ice for 10 min and then centrifuged at 11,500 × g for 15 min. After centrifugation, 500 μL of the sample solution below the mineral oil was transferred to a clean 1.5 mL Eppendorf tube and the pH of the solution was adjusted to between 4.1 and 4.5 with 10% v/v ammonium hydroxide (Sigma-Aldrich) if required. Then 10 μL of the sample were aliquoted in triplicate into a 96-well plate (Corning) containing 140 μL of pH 4.2-ammonium acetate solution. Varying concentrations of Alizarin red solution diluted in the same ammonium acetate solution were used as standards and the ammonium acetate solution was used as the blank. The plate was read at 405 nm on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). For this assay, the calcium content of each sample was calculated with SoftMax Pro 4.8 software (Molecular Devices), using a 1:1 stoichiometric ratio for the Alizarin red-calcium complex (29).

Magnetic resonance microscopy

Collagen strips for each time point (3, 6, 9, 13, 15, 18, and 24 days) along with control strips were analyzed by MRM. For the MRM experiment, strips were sandwiched between two glass slides (width = 12 mm, height = 75 mm)